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PHYTOCHEMICAL, ANTIMICROBIAL AND ACUTE TOXICITY STUDIES OF *Ipomoea asarifolia* LEAVES (CONVOLVULACEAE)

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ABSTRACT
Plants have provided sources for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being. The leaves of Ipomoea asarifolia were ground in to powder followed by successive extraction (methanol and water) and then screened for phytochemicals using the standard methods. Antimicrobial activities of the crude extract against clinical isolates were investigated using agar well diffusion, broth dilution methods. The antibiotic susceptibility profiles of the microbial isolates to standard antifungals and antibacterial such as fluconazole and ciprofloxacin respectively were determined using standard methods. Phytochemical screening revealed the presence of tannins, saponins, flavonoids, triterpenes, phenols and alkaloids. The methanol extract of the plant leaves produced the moderate antifungal and antibacterial activity when compared with the standard drug. The antimicrobial sensitivity results revealed that methanolic extract had promising antibacterial and fungal activity at highest concentration 500 mg/ml. Shigella sp. was found to be the most susceptible clinical isolate at 500mg/ml with inhibition zones of 16 mm, Candida kfyer was next most susceptible isolate to methanol extract of the leaves with inhibition zone of 15 mm. Klebsiella sp., Escherichia coli and Shigella sp. showed MIC and MBC of 31.25 mg/ml and 62.5 respectively. The LD_{50} of Ipomoea asarifolia was found to be greater than 5000 mg /kg and could be considered safe for consumption. Further investigations should be carried out to isolate pure compounds and determine the mechanisms of action of the plant.
Keywords: Ipomoea asarifolia, fluconazole and ciprofloxacin, Antimicrobial, Phytochemical screening

INTRODUCTION

The traditional treatment and control of diseases by the use of available medicinal plants by rural communities will continue to play significant roles in medical health care implementation in the developing countries (Ekundayo *et al.,* 2011). The use of synthetic medicines as therapeutic agents however is limited, due to various challenges such as poor drug solubility, stability, adsorption and high toxicity. In addition, some of these drugs are expensive and generally unavailable to citizens of developing countries, especially those residing in the rural areas (Sule *et al*., 2011). The shortfalls in the use of chemotherapeutic agents as control agents of bacterial diseases, further encourages the use of plants as a form of alternative medicine.

In Nigeria it is commonly known as 'Duman-kaada', 'Duman-raafi' or 'Duman-kadu' in Hausa (northern Nigeria), 'Gboro-ayaba', 'Ododo-oko' and 'Ododo-amu' in Yoruba (south west-Nigeria) (Jegede *et al.,* 2009) and it have been used for the treatment of various ailments such as diabetes, neuralgia, stomach ache and arthritic pain, dysmenorrhea, guinea worm sores and liver diseases (Akindale *et al.,* 2015). Hausa/fulani people from northern Nigeria uses this herb to treat feverish chills and rheumatic pains, guinea-worm sores, syphilis (Maryam and Mericli, 2017).

Ipomoea asarifolia is commonly known as ginger-leaf morning-glory. Its stems are hollow and possesses a large red-purple/pinkish-purple flowers that bears same number (five) of anthers, filaments and a glabrous sepals with superior ovaries, pollen grains which is 32.3-34.2µm in diameter is circular oblate spheroidal, radially symmetrical (Jayeola and Oladunjoye, 2012). *Ipomoea asarifolia* is popularly known as

salsa or salsa-brava in Brazil and the decoction of its leaves are used in ethnomedicine to treat diverse inflammatory disorders such of scabies and dermatitis and for the treatment of syphilis, skin ulcers and external wounds (Furtado *et al.,* 2016).

In Senegal, *I. asarifolia* is used traditionally to treat various gynecological ailments (including urinary problems during pregnancy, hemorrhage, abortificient and ecbolic), also the plant is used to treat arthritis pain, neuralgia, headache, wound dressing, opthalmia (Aliyu *et al.,* 2011).The indiscriminate use of herbal medicines including *Ipomoea asarifolia* plant in our communities despite its claimed efficacy still poses a lot of medical challenges on the users and therefore its phytochemical constituents, antimicrobial activities and its safety profile need to be evaluated for scientific proof.

MATERIALS AND METHODS

Collection of Clinical Specimen

Ethical clearance with the number MOH/Off/797/T.I/645 was obtained from the ethical committee of Kano State Hospital Management Board, Kano State Ministry of Health for all the samples collected. Thus patients were asked to clean their external genitalia with disinfectant and their midstream urine was collected in a sterilized cap. Samples were kept in an ice bag and transported to microbiological laboratory.

Urine culturing

Urine samples were cultured on Nutrient agar, blood agar and MacConkey agar media and incubated over night at 37°C. Significant growth was evaluated as ≥ 105 colony-forming units CFU/mL of midstream urine (Karzan*et al.,* 2017).

Culture characteristics

Each of the color, size, elevation, margins and texture of colonies were screened. The morphological different colonies on MacConkey agar, nutrient agar and blood agar were sub-cultured into nutrient agar medium, in order to purify the isolated bacteria from each patient urine specimen (Karzan*et al.,* 2017).

Microscopic examination

Pure isolates were examined microscopically, on the basis of their cell wall composition and presence of capsule (Karzan*et al.,* 2017).

Microbiological analysis

According to Gram staining technique, isolates were cultured on numerous selective and differential media to find out their color, colony morphology and ability of fermentation (Karzan*et al.,* 2017).

Isolation of Bacteria Species

The specimens were cultured on sterile blood agar, chocolate agar and Mac-conkey agar plates at 37°C for 24 h in an incubator. Discrete colonies were picked based on their morphology and further sub-cultured on blood agar and chocolate agar to obtain pure strains. The isolated colonies were Gram stained and based on their Gram reactions were inoculated on different selective media — mannitol salt agar, cetrimide agar, eosin methylene blue agar. Different biochemical tests were conducted (catalase, coagulase, and oxidase tests). All the isolates that grew on selected agar media were then placed on nutrient agar and chocolate agar slants and maintained in a refrigerator at 4°C (Cheesbrough, 2010).

Identification and Characterization of Test Organism using Rapid Test Kits

Identification and characterization of the bacteria was carried out using Microgen Identification Kit (XYZ). The test was performed according to the manufacturer's specifications (API biomerieux). It was performed by adding saline suspension of the test organisms to each of the wells, and appropriate wells (1, 2, 3 and 9) were overlaid with sterile paraffin oil. After overnight incubation (18-24 hours) at 37°C, suitable reagents such as Nitrate A and B, Kovacs, Typtophandeaminase (TDA), Voges-proskauer (VPI and II) were added to wells 8, 10 and 12 for additional tests and colour changes of the different tests recorded. The results were converted into four to eight digits codes depending on the organisms being tested and interpreted using the Microgen Identification Software Package (MID-60) (Sylvester, 2016).

Collection and Identification of Plant Materials

The leaves of *Ipomoea asarifolia* were collected from local farm at Kumbotso Local government area, Kano state, Nigeria. The plant was identified and authenticated in the herbarium of the Plant Biology Department of Bayero University, Kano, Kano State, Nigeria and a voucher specimen number was deposited.

Preparation of Plant extracts

The leaves of the *Ipomoea asarifolia* were cleaned, air dried and ground to coarse powder using grinding machine. The powder was stored in air tight containers for further use. One hundred grams (100 g) each of the powdered leaves was soaked into 1000ml each of methanol and distilled water. The mixtures were allowed to stand for 3 days at room temperature (28 ±2ºC) with hourly agitations. Each extract was sieved through a muslin cloth, filtered through a Whatman (No.1) filter paper, poured unto a clean evaporating dish and placed on a water bath at 50ºC until all the solvent evaporated.

Qualitative Phytochemical screening of Aqueous and Methanolic extract of *Ipomoea asarifolia* Leaves

The plant extracts were subjected to phytochemical screening in order to identify the phytochemical constituents of the plant using the methods described below.

Tests for carbohydrates

Molish's (General) Test for Carbohydrates: To 1 ml of the filtrate, 1 ml of Molish's reagent was added in a test tube, followed by 1 ml of concentrated sulphuric acid down the test tube to form a lower layer. A reddish colour at the interfacial ring indicates the presence of carbohydrate (Evans, 2009).

Tests for Saponins

Frothing test: About 10ml of distilled water was added to a portion of the extract and was shaken vigorously for 30seconds. The tube was allowed to stand in a vertical position and was observed for 30mins. A honeycomb froth that persists for 10-15mins indicates presence of saponins (Evans, 2009).

Test for Flavonoids

Shinoda Test: A portion of the extract was dissolved in 1-2ml of 50% methanol in the presence of heated metallic magnesium chips and a few drops of concentrated hydrochloric acid were added. Appearance of red color indicates the presence of flavonoids (Evan, 2009).

Test for Alkaloids

Wagner's Test: Few drops of Wagner's reagent was added into a portion of the extract, whitish precipitate indicates the presence of alkaloids (Evans, 2009).

Test for Steroids and Triterpenes

Liebermann-Burchard's test:

Equal volumes of acetic acid anhydride was added to the portion of the extract and mixed gently. Concentrated sulphuric acid (1 ml) was added down the side of the test tube to form a lower layer. A colour change observed immediately and later indicates the presence of steroid and triterpenes. Red, pink or purple colour indicates the presence of triterpenes while blue or blue green indicates steroids (Evans, 2009).

Test for Cardiac Glycosides

Kella-killiani's test:

A portion of the extract was dissolved in 1ml of glacial acetic acid containing traces of ferric chloride solution. This was then transferred into a dry test tube and 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer at the bottom. Interphase for purple-brown ring was carefully observed, this indicates the presence of deoxy sugars and pale green colour in the upper acetic acid layer indicates the presence of cardiac glycosides (Evans, 2009).

Test for Tannins

Ferric chloride test:

Exactly 3-5 drops of ferric chloride solution was added to the portion of the extract. A greenish black precipitate indicates the presence of condensed tannins while hydrolysable tannins give a blue or brownish blue precipitates (Evans, 2009).

Test for Anthraquinones

Borntrager's test:

Exactly 5ml of chloroform was added to the portion of the extract in a dry test tube and shaken for at least 5mins. This was filtered, and the filtrate shaken with equal volume of 10% ammonium solution, bright pink colour in the aqueous upper layer indicates the presence of free anthraquinones (Evans, 2009).

Quantitative Determination of Phytochemical Contents of *Ipomoea asarifolia* Leaves

Determination of Alkaloids

About 5g of the sample was weighed into a 250ml beaker and 200ml of 10% acetic acid in ethanol were added and covered and allowed to stand for 4hours. This was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation is completed. The whole solution was allowed to settle, and the precipitates were collected, washed with dilute ammonium hydroxide solution and then filtered. The residue was the alkaloids, which was then dried and weighed (Haborne, 1973).

Determination of Flavonoids

About 10g of the plant sample was extracted repeatedly with 100ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter upper No. 42 (125mm). The filtrate was later be transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight (Bohm and Kocipal – Abyazan, 1994).

Determination of Saponins

The method of Obadoni and Ochuko (2001) was used. Out of the ground samples 10g was weighed for each into a conical flask and 100ml of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4hours with continuous stirring at about 55oC. The mixture was filtered and the residue re-extracted with another 200ml, 20% ethanol. The combined extracts were reduced to 40ml over water bath at about 90^{o}C. The concentrate was transferred into a 250ml separatory funnel and

20ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60ml of n – butanol was added. The combined *n*-butanol extracts were washed twice with 10ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight. The saponins content was calculated as percentage.

Determination of Tannins

About 500mg of each sample was weighed into a 50ml plastic bottle and 50ml of distilled water was added and shaken for 1hour on a mechanical shaker. This was filtered into a 50ml volumetric flask and made up to the mark. Then 5ml of the filtrate was pipetted out into a test tube and mixed with 2ml of 0.1M $FeCl_3$ in 0.1M HCl and 0.008M potassium ferro-cyanide. The absorbance was measure at 120mm within 10mins (Van-Burden and Robinson, 1981).

Determination of Total Phenols

The fat free sample was boiled with 50ml of ether for 15minutes. About 5ml of the extract was pipetted into a 50ml flask, and then 10ml of distilled water was added. About 2ml of ammonium hydroxide solution and 5ml of concentrated amyl alcohol were added. The sample was made up to mark and allowed to react for about 30 minutes for colour development. This was measured at 505nm.

Antimicrobial Susceptibility Test

Preparation of Extract Concentration

This was carried out according to the method described by Srinivasan *et al.,* (2009). Stock solution of the plant extracts were prepared by adding 0.5g of each crude plant extract in 1ml dimethyl sulphuroxide (DMSO). From each of the stock solutions, 500mg/ml, 250mg/ml, 125mg/ml and 62.5mg/ml concentrations were prepared using Two-fold serial dilution method (Srinivasan *et al.,* 2009).

Standardization of bacterial Inoculum.

Using inoculum loop, over-night grown agar culture (bacteria and fungi) was transferred into a test tube containing normal saline until the turbidity of the suspension matched the turbidity of the 0.5 McFarland Standard as described by the National committee for clinical laboratory standard (NCCLS, 2008).

Susceptibility Test of Bacterial and Fungal isolates to Different Concentrations of the Extracts

The antimicrobial activity of *Ipomoea asarifolia* crude extract (Methanol and aqueous) against *Klebsiella sp., Staphylococcus aureus, Shigella sp., Escherichia coli, Candida glabrata, Candida tropicalis, Candida krusei, Candida kfyer* and *Candida albicans* were evaluated using agar well diffusion method of susceptibility test (Srinivasan *et. al.,* 2009*)*. Mueller-Hinton agar and Sabouraud Dextrose agar plates were inoculated with 0.1ml of standardized inoculum of each bacterium and fungus respectively (in triplicates) using 0.1ml pipette and spread uniformly with sterile swab sticks. Three wells of 6mm size were made with sterile cork borer (6 mm) into the inoculated agar plates. Using micropipette, 0.1ml volume of the various concentrations; 500mg/ml, 250mg/ml, 125mg/ml and 62.5mg/ml each of the crude extracts were dispensed into wells of inoculated plates. . DMSO was used as negative control. Commercially available standard antibiotic, ciprofloxacin and fluconazole were used as positive control parallel with the extracts. The prepared plates were then left at room temperature (37 ºC) for 10minutes, allowing the diffusion of the extracts into theincubation at 37 ºC for 24 hrsin an incubator. The diameter of inhibition zones (DIZ) were measured and expressed.

Determination of Minimum Inhibitory Concentration (MIC)

The method used was the tube dilution method (Adesokan *et al*., 2007). Thus, the plant extracts were serially diluted from 500 mg/ml solution to obtain varying concentration. The concentrations were; 250 mg/ml, 125 mg/ml, 62.5mg/ml, and 31.25 mg/ml. Doubling dilutions of the extract were incorporated in Muller Hinton broth (Oxoid, UK), and then inoculated with 0.1ml each of standardized suspension of the test organisms into the various test tube containing varying concentrations. Another set of test tubes containing only Mueller Hinton broth were used as negative control, and another test tube containing Mueller Hinton broth and test organisms were used as positive control.All the test tubes and controls were then incubated at 37 ^{o}Cfor 24hrs. After incubation period, the presence or absence of growth on each tube was observed, loop full from each tube was further sub cultured onto nutrient agar to confirm whether the bacterial growth was inhibited.

Determination of Minimum Bactericidal Concentration (MBC)

The MBC was determined by collecting 1ml of broth culture from the tubes used for the MIC determination and subculturing into fresh solid nutrient agar plates.

The plates were incubated at 37 ^{0}C for 24 h. The least concentration that did not show any growth after incubation was regarded as the MBC (Adesokan *et al.*, 2007).

Acute toxicity studies of methanol extract of *Ipomoea asarifolia* Leaves

Lethal Dose (LD_{50}) Determination

The method of Lorke (1983) was employed. Thus, the phase I involved the oral administration of three different doses of 10, 100 and 1,000 mg/kg of the crude extract, to three different groups of three adult wister albino rats. In a fourth group, three adult male wister albino rats were administered with equivalent/volume of distilled water to serve as control. All the animals were orally administered with the extracts using a curved needle to which acatheter had been fixed. The animals were monitored closely every 30 minutes for the first 3 hours after administration of the crude extracts, and then hourly for the next 6hours for any adverse effects. Then the animals were left for 72 hours for further observation.

When no death occurred, the phase II was employed, only one animal was required in each group. Groups 1–4, animals were orally given 1,500, 2,200, 3250 and 5,000mg/kg dose levels of the crude extract. All the animals were left for observation as in stage one.

RESULTS

Phytochemicals which include alkaloids, flavonoids, saponins, tannins, glycosides, carbohydrates and triterpenes were detected in both aqueous and methanolic extracts while steroid is absent. Anthraquinones were detected in the methanolic extract but absent in aqueous extract of *I. asarifolia.*

Table1. Qualitative Phytochemical Constituents of the Aqueous and Methanolic extracts of *Ipomoea asarifolia* Leaves

Phytochemicals	Extracts	
	Aqueous	Methanolic extract
Alkaloid	+	+
Flavonoid	+	+
Saponins	+	+
Cardiac glycoside	+	+
Tannins	+	+
Steroid	-	-
Triterpenes	+	+
Phenol	-	+
Anthraquinones	-	+
Carbohydrate	+	+

Key: + Present, - Absent

The flavonoids (148.0 mg/g) was the highest phytochemical detected in the plant while the lowest was saponins and saponins (5.0 mg/g).

Table 2. Quantitative Phytochemical Contents of *Ipomoea asarifolia* Leaves

Metabolite	Quantity (mg/g)
Alkaloids	130.0±0.33
Flavonoids	148.0±0.48
Saponins	5.00±0.0.58
Tannins	42.80±0.21
Phenols	8.00 ±0.33

Table 3. Antimicrobial activity of methanol extract of *Ipomoea asarifolia* Leaves

Clinical isolates	**Concentration/Diameter zone of inhibition(mm)**						**MIC**	**MBC**
	500	250	125	62.5	CPR/FLC	DMSO		
S. aureus	13	11	8	6	41/*	6	-	-
E. coli	14	11	9	7	40/*	6	31.25	62.5
Klebsiella sp.	14	11	9	7	36/*	6	31.25	62.5
Shigella sp.	16	14	11	9	38/*	6	31.25	62.5
C. albicans	13	11	10	6	*/40	6	-	-
C. glabrata	14	11	9	6	*/38	6	-	-
C. krusei	13	10	8	6	*/37	6	-	-
C. tropicalis	14	11	9	6	*/38	6	-	-
C. kfyer	15	12	10	6	*/39	6	-	-

Key: * = Not test CPR (Ciprofloxacin was only used against bacterial isolates), FLC (Fluconazole was used against fungal isolates).

From the results of the acute toxicity studies (Table 4), no death was recorded in the first phase. In the second phase, doses of 1500, 2250, 3250 and 5000mg/kg were used and no death was also recorded. The oral median lethal dose (LD_{50}) for the methanolic leaf-extract of

Ipomoea asarifolia was therefore estimated to be greater than 5000mg/kg and no sign of behavioural changes were also observed.

Table 4. Acute toxicity studies of methanolic extract of *Ipomoea asarifolia* Leaves

Treatment	Group	Number of Animals	Dose (mg/kg)	Mortality recorded after 24hrs
Phase I	I	3	10	0/3
	II	3	100	0/3
	III	3	1000	0/3
Phase II	I	1	1500	0/1
	II	1	2250	0/1
	III	1	3250	0/1
	IV	1	5000	0/1

DISCUSSION

Phytochemical investigation of the leaf extract of *Ipomoea asarifolia* led to the identification of saponins, anthraquinones, phenols, tannin, alkaloid flavonoids e.g. rutin (De Souza Lima *el al.,* 2014). According to Jegede *et al,* (2009) Cardiac glycosides, flavonoid, volatile oil and terpenes were found to be absent in the leaf extract of *I. asarifolia*. The therapeutic value of medicinal plants lies in the various chemical constituents in it. The bioactivity of plant extracts is attributed to its phytochemical constituents (Farooq, 2014). The phytochemical analysis showed that methanol leaf extract tested positive to saponins, carbohydrates, alkaloids, glycosides, proteins, triterpenes and tannins. Due to the presence of saponins in the extract, it was found that saponins has relationship with sex hormone involved in controlling the onset of labor in women and the subsequent release of milk (Okwu and Okwu, 2004).

The result from Table 3 shows that, methanol extract at 500 mg/ml showed maximum antimicrobial activity against *Shigella sp.*(16 mm)followed by *Candida kfyer* (15 mm) and *E. coli., C. glabrata, C. tropicalis* and *Klebsiella sp.* with 14 mm each. These results goes together with the study conducted by Junaid (2008) that the aqueous root extract showed a relatively moderate zone of inhibition on *E. coli* and *S. aureus* (15 mm and 14 mm).The antibacterial test results presented in Table 3 showed good activity against tested bacteria with low antifungal activity. The methanolic extract exhibited considerable level of inhibition against the entire test organism compared to the standard drug at 500 mg/ml. This is suggestive of the presence of some compounds or groups in the extract with similar mechanism of action to that of the standard drug used. In addition, microorganisms show variable sensitivity to chemical substances related to different resistance levels between strains (Sharma *et al.,* 2012).The antimicrobial effects of *Ipomoea asarifolia* had been previously studied and reported that the extract displayed potent antimicrobial activity against *S. aureus, P. aeruginosa, Bacillus subtilis, Bacillus cereus, E. coli* and host of other bacteria and fungi, giving inhibitory concentration as low as 0.5ml (Arekemase *et al.,* 2011) which confirms the potency of this plant in treating human infections. The MIC and MBC assay procedures are frequently used to evaluate some diverse agents such as antibiotics, antiseptics, disinfectants and chemotherapeutic agents (Andrews, 2001). In this study, the MIC and MBC values of both *Klebsiella sp., Escherichia coli and Shigella sp.* with methanolic extracts of *Ipomoea asarifolia* indicates significant bactericidal activities. This implies the strong efficacy of the extracts as stated by Arekemase (2011) that the constituents of the leaves of *Ipomoea asarifolia* contains phenols, flavonoids and some secondary metabolites that are very useful in antimicrobial activity.

Acute toxicity studies of *Ipomoea asarifolia* leaves was performed using the Lorke method and using the limit dose test of Up and Down method. With careful observations of experimental animals from the first 30 minutes up to the 48 hrs, it was revealed that there were no deaths and any sign of toxicity such as loss or increase in weight, tiredness, abdominal constrict convulsion, hyperactive, weakness, diarrhea or increased diuresis within the short and long term effect in rats dosed with 5000 mg/kg body weight of the *Ipomoea asarifolia* extracts (aqueous and methanol). The outcome of the study of Alhassan *et al.,* (2014) gave an LD50 of 2000 mg/kg and this guided our choice of dose used (5000 mg/kg). The LD50 was found to be greater than 5000 mg/kg body weight orally, and this suggested that the extract has low acute toxicity when administered orally. This may be attributed to the incomplete absorption brought about by inherent factors limiting absorption in the gastro intestinal tract (Dennis, 1984). The present study agrees with

the work done by Prasanth *et al*., (2015) Adesegun *et al.,* (2016). Bruce, (2006) reported that any substance with LD_{50} estimated to be greater than 2000-5000 mg/kg body weight given orally could be considered to be of low toxicity and safe. The very high LD_{50} observed is not a conclusive finding about the safety of the extracts of *Ipomoea asarifolia*, higher doses could be tested for better understanding of its effects if use for a long period of time and for proper recommendation on its future utilization (Ogbonnia *et al.,* 2011).

CONCLUSION

Ipomoea asarifolia extracts have some secondary metabolites namely alkaloids, tannins, flavonoids, cardiac glycosides and saponins. These contribute in the effect of the plant. The Acute toxicity (LD_{50}) of the leaf extracts of *Ipomoea asarifolia* (Aqueous and Methanol) was found to be greater than 5000 mg /kg and is considered safe for use. The extracts have antimicrobial effects that are concentration dependent. This supports the basis for the use of this plant traditionally for different ailments. The effects are probably due to phenolic compounds, flavonoids, tannins and some elements in the plant.

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